PLANT TISSUE CULTURES FROM A HORMONE POINT OF VIEW

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A botanist, Haberlandt,[1] first pointed out the possibilities of the culture of isolated tissues. He suggested that not only could the potentialities of individual cells be determined, but that also some insight might be gained as to the reciprocal influences of tissues upon one another, that is, as to "correlation." Haberlandt's many experiments with plant cells did not succeed. Harrison, Burrows and Carrel, using animal tissue, were able to realize Haberlandt's ideal, and from the more recent work of Fischer, Ephrussi, Gaillard and Chambers, it is evident that many different kinds of problems can be attacked by the method of tissue culture. Since the time of Haberlandt there have been repeated attempts to cultivate plant tissue in artificial media, or at least *in vitro*. Embryonic tissue has been grown under many conditions. Thus Hannig[2] grew immature embryos in artificial media and eventually raised mature plants from them. LaRue[3] has recently cultivated fragments of embryos and has obtained mature plants from such fragments. It should be clear, however, that the concept of "tissue culture" does not embrace such experiments. The embryos of Hannig and others have always differentiated into more or less normal complexes of tissue even though grown on artificial media. The embryonic fragments of LaRue have similarly differentiated, and are, in fact, typical examples of "regeneration" in the sense of Loeb. Isolated root tips were first cultivated *in vitro* by Robbins[4] and by Kotte,[5] but to White[6] is due the credit for having worked out the cultural conditions capable of sustaining the indefinite growth of these organs. In this case again, as is well shown in the recent work of Robbins, Bartley and White,[7] the root meristem continues to differentiate in a comparatively normal fashion. Cultures of the various component tissue of the root have not as yet been possible. In fact, cultivation of isolated plant tissue has been accomplished only by Schmucker[8] with palisade cells, but his results cannot as yet be confirmed.

This signal lack of success in the cultivation of isolated plant tissues is undoubtedly in part due to the use of unsuitable tissue. On the other hand it is also certainly due in part to unsuitability of the media employed. Because of the work on auxin we are now beginning to realize the great part special chemical substances or "hormones" play in plant growth and development, and it seems probable that such substances must be present in any medium in which plant tissue is to be successfully cultivated. We cannot, however, supply these substances as simply to plant cultures as to

animal, since they are not readily available in embryonic extract. Experience has shown that extract of plant embryos is in general without effect or actually toxic for the growth of excised plant tissue. White has shown, however, that there is a substance (or substances) present in minute amounts in yeast which is essential for the continued growth of excised roots. That analogous substances are necessary for the growth of other organs and tissues, and that these substances may also be derived from outside sources, seems a fair inference. The objectives of the present work have therefore been the separation and purification of special substances or hormones which control growth and development of plant tissues and organs, and the perfection with the aid of such substances of true *tissue cultures*. Only a portion of this work will be reported here, and the remainder will be discussed in greater detail elsewhere.

If a young string-bean pod be split lengthwise along the sutures and the immature seeds removed, a series of "cups" lined with small iso-diametric parenchymous cells is exposed. The parenchymous lining of the cup is very sensitive to injury and responds to a prick by a hypertrophic outgrowth of dividing and enlarging cells. A similar but larger outgrowth follows in response to certain tissue extracts, as first described by Wehnelt.[9] If small drops of water are placed in the tissue cup no reaction occurs.[10] Similarly, auxin is without effect except in concentrations sufficiently high to be toxic, when a small reaction occurs (see also Jost[11]). Tissue extracts, however, either of the bean or of other plant organs, particularly green parts, cause a "wart" like protuberance of the parenchymous tissue. This outgrowth is limited to the area directly under the drop of tissue extract, and may be as much as 1.5 mm. high. Cross-sections of the wart show that it consists of cells elongated at right angles to the surface of the cup. Cell divisions may also be found (see Jost[11]), their number depending on the variety of bean used. Thus golden wax and valentine varieties gave no cell divisions, Kentucky wonder number 1 gave a few, and Kentucky wonder number 2 gave many cell divisions.

Standard conditions for the "bean test" have been established, and it has been possible to perform semi-quantitative tests for the factor (or factors) in question by determination of the dilution in which a given preparation is just active. In this way the activity of a solution may be estimated to about ± 3 times. The following chemical properties of the factor involved, a few of which were previously reported by Wehnelt, have been established:

1. Soluble in water, 75% alcohol.
2. Insoluble in ether, chloroform, butyl alcohol, absolute alcohol.
3. Stable to H_2O_2 in both acid and alkaline solution.
4. Relatively heat stable in both acid and alkaline solution.
5. Adsorbable from water by charcoal, elutable in 75% alcohol.

6. Precipitatable by alkaloidal reagents (phosphotungstic acid, picric acid).

A partial purification of the factor is possible on the basis of the properties enumerated above, and this purification is being continued. It would be of interest to know whether or not the bean reaction is due to one factor influencing both cell division and cell elongation, or to two or more separate substances. With this in view, pantothenic acid, a "growth hormone" for certain strains of yeast, and kindly supplied by Prof. Roger Williams, was tried in the bean test. It was completely without activity over a wide range of concentrations. Similarly vitamines B_1 and B_2, shown by Schopfer[12] to be growth substances of Phycomyces, do not come in question here since they have chemical properties different from those enumerated above. The conclusion of Dagys[13] that the growth hormones of the lower and the higher plants are closely related would seem open to some question. One may also draw the conclusion that the auxins are not the only cell elongation hormones of the higher plants, since we have here cell elongation due to a substance of quite a different type (insoluble in ether and chloroform).

The fact that the parenchymous cells of the bean pod respond actively to this extract *in vivo* suggests that such cells might be suitable for cultivation *in vitro*. Accordingly a large number of experiments on their cultivation in liquid media have been made. Of the various solutions, used as a base for the medium, the best thus far has been found to be a somewhat modified White's[6] solution; although this is in all probability not optimal. The solution was made up as follows:

$Ca(NO_3)_2$	230 mg.	KH_2PO_4	12 mg.
$MgSO_4$	35 mg.	$Fe_2(SO_4)_3$	2 mg.
KNO_3	80 mg.	sucrose	20 gm.
KCl	65 mg.	water	1 liter

Tissue extract for the experiments reported here were made by extracting 250 gm. of fresh beans with 250 cc. of 75% alcohol for 8 hours, evaporation of the alcohol, filtering and dilution of the filtrate to a total volume of 200 cc. This extract was then diluted with an equal volume of the above culture solutions which had been made up to twice the final concentration.

Cubic pieces of tissue approximately 1 mm. on each side were removed under sterile conditions from bean pods and placed in petri dishes with 10 cc. of the above medium. The petri dishes were then left in the dark for 6 days. At the end of this period control cultures to which no extract had been added showed no growth whatsoever. If bean extract had been added a very striking growth of the parenchymous tissue took place; in some experiments an increase of volume of as much as 40 times. The originally cubical tissue fragment rounded out as it enlarged and assumed a spherical shape. The enlargement depended, in the main, upon elongation of the peripheral cells. At the base of this zone of greatly elongated cells was found

a "meristematic" zone, particularly in cultures of Kentucky wonder number 2. Superficially these cultures resembled strikingly the cultures of fibroblasts *in vitro*. In reality, of course, the cells at the periphery were "migrating" only by their growth and not by any independent active movement.

At the end of six days new fragments approximately 1 mm. on each side were cut from the central portion of the original cultures and placed in fresh medium. These "subcultures" grew as did the original cultures. Subcultures were continued, each time fragments being taken from the center of the old culture. After the first two cultures, however, the growth steadily declined until it became practically 0, as is shown in table 1.

TABLE 1

No. of subculture	0	1	2	3	4	5	6	7
Av. diam., mm.	1	4.5 ± 0.6	4.5 ± 0.7	3.3 ± 0.3	2.8 ± 0.3	1.8 ± 0.3	1.5 ± 0.3	1.3 ± 0.2

It was at first supposed that this decline might be due to the exhaustion of some other essential factor originally present in the tissue. The addition of other substances to the later cultures, as well as to the culture during its entire history, was therefore attempted. Peptone, yeast extract, heteroauxin and malt extract were tried in this way, each over a considerable range of concentrations, but no substantial improvement of the later cultures was found. It seems more likely that there is a cumulative toxic effect of the crude preparation employed, but a test of this hypothesis must await further purification of the active principle.

The chief interest of the cultures described here is that they represent a somewhat closer approach to true plant *tissue* cultures than do other attempts thus far reported, even though they are not, as yet, capable of indefinite subculture. They show, in the first place, both cell division and cell enlargement, and not merely cell enlargement as did the cultures of Haberlandt, Bobilioff-Preisser[14] and others. In the second place, parenchymous tissue in this case continues to grow as parenchymous tissue, and there is no differentiation into root and shoot as in the cultures of LaRue, or into vascular tissue as in the root cultures of White. It is necessary to note, however, that the present cultures are not completely without a progressive differentiation, since the greatly enlarged cells of the periphery are incapable of further division, and subcultures must be made from the central portion of smaller, dividing cells.

[1] Haberlandt, G., *Sitzungsb. Akad. Wiss. Wien, Math. Natur. Kl.*, **3**, 69 (1902).
[2] Hannig, E., *Bot. Zeitung*, **62**, 45 (1904).
[3] LaRue, C., *Proc. Nat. Acad. Sci.*, **22**, 201 (1936).
[4] Robbins, W., *Bot. Gaz.*, **73**, 376 (1922).
[5] Kotte, W., *Beitr. z. allg. Bot.*, **2**, 413 (1922).

[6] White, P., *Plant Physiol.*, **9**, 585 (1934).
[7] Robbins, W., Bartley, M., and White, V., *Bot. Gaz.*, **97**, 554 (1936).
[8] Schmucker, Th., *Planta*, **9**, 339 (1930).
[9] Wehnelt, B., *Jahrb. wiss. Bot.*, **66**, 773 (1927).
[10] Wehnelt experienced difficulty in that pure water sometimes caused a reaction. If the pods are allowed to wilt slightly before use this difficulty can be avoided.
[11] Jost, L., *Ber. d. d. bot. Gesell.*, **53**, 733 (1935).
[12] Schopfer, W., *Ibid.*, **52**, 308 (1934).
[13] Dagys, J., *Protoplasma*, **24**, 14 (1935).
[14] Bobilioff-Preisser, W., *Beih. z. bot. Zentralbl.*, **33**, 248 (1917).

'INHIBITION OF REINFORCEMENT' AND PHENOMENA OF EXPERIMENTAL EXTINCTION

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In the literature on conditioned reflexes, two contrasted varieties of curves of experimental extinction have been described. The first type, illustrated in the experiments of Pavlov,[1] Kleitman and Crisler,[2] Switzer[3] and others, is characterized by a continuous decline in the magnitude of the conditioned response on successive unreinforced elicitations. The decline is rapid at first, then more and more gradual. The second type, reported by Switzer,[4] Scott,[5] Hudgins[6] and Hilgard and Marquis,[7] shows a larger response on the second or third extinction trial than on the first, demonstrating the initial rise characteristic of these curves. Hull,[8] in his summary of studies of conditioned reflexes, points out that "as yet the experimental conditions which bring about these two types of curve have not been determined" (p. 438).

The initial rise in the extinction curves of the second variety has been attributed to a "practice effect" or "improvement through use." This fails to explain why the rise is not uniformly observed. Research by the writer[9] on the irradiation of conditioned responses has suggested an explanation based upon the experimental conditions of the reinforcement process. There appears to be a negative adaptation to continuous reinforcement, an effect which may be labeled, for descriptive purposes, the 'inhibition of reinforcement.'[10] Following protracted reinforcement the omission of the unconditioned stimulus might be expected to produce 'disinhibition,' resulting in an augmentation of response on the second or third extinction trial. This explanation for the rise in their extinction curves has been recently proposed by Hilgard and Marquis:[7] "The first extinction trial is followed by altered conditions. . . This unexpected